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(54) Title: REFOLDING TRANSFORMING GROWTH FACTOR BETA FAMILY PROTEINS

(57) Abstract: Compositions and methods for folding proteins belonging to the transforming growth factor beta superfamily are disclosed. The compositions and methods allow for the folding of such proteins when produced in an expression system that does not yield a properly folded, biologically active product.



WO 2006/023782 A2

## REFOLDING TRANSFORMING GROWTH FACTOR BETA FAMILY PROTEINS

Cross Reference To Related Applications

This application claims priority from provisional application number 60/602,825,  
5 filed August 19, 2004. The entire content of the prior application is incorporated herein  
by reference in its entirety.

Technical Field

The invention relates to compositions and methods for refolding proteins  
10 belonging to the transforming growth factor beta superfamily.

Background

Neublastin, also known as Artemin and Enovin, is a 24-kDa homodimeric  
secreted protein that promotes the survival of neurons of the peripheral and central  
15 nervous system such as dopaminergic neurons (Baudet et al., 2000, *Development*,  
127:4335; Roseblad et al., 2000, *Mol. Cell Neurosci.*, 15(2):199; GenBank™ AF120274).  
The gene encoding Neublastin has been cloned and sequenced (Roseblad et al., 2000,  
*Mol. Cell Neurosci.*, 15(2):199; Baloh et al., *Neuron*, 21:1291).

Neublastin is a member of the glial cell line-derived neurotrophic factor (GDNF)  
20 ligand family. At the cellular level, GDNF members activate the receptor tyrosine  
kinase, RET. RET associates with a co-receptor, GDNF family receptor  $\alpha$  (GFR $\alpha$ ), a  
glycosylphosphatidyl inositol (GPI) linked membrane protein that provides ligand  
specificity for RET. Four GFR $\alpha$ 's are known (GFR $\alpha$ 1-4). Neublastin binds to GFR $\alpha$ 3  
together with RET forming a ternary signaling complex (Baudet et al. 2000,  
25 *Development*, 127:4335; Baloh et al., 1998, *Neuron*, 21:1291), which is localized  
predominantly on nociceptive sensory neurons (Orozco et al., 2001, *Eur. J. Neurosci.*,  
13(11):2177). These neurons detect pain and injury. Thus, Neublastin has clinical  
application in the general treatment of neuropathy and more specifically in the treatment  
of neuropathic pain.

Neublastin and the other GDNF family members are members of the transforming growth factor beta (TGF beta) superfamily and thus, are characterized by the presence of seven conserved cysteine residues with similar spacing which form the structure of a cysteine knot (Saarma, 1999, *Microsc. Res. Tech.*, 45:292). Each monomer contains two  
5 disulfide bonds that form a closed loop structure encircling the third disulfide to form a tight knot structure. The seventh cysteine contained within each monomer forms an intermolecular disulfide bond, covalently linking the monomers to form the final dimer product (Rattenholl et al 2000, *J. Mol. Biol.*, 305:523).

TGF beta family members are synthesized as pre pro proteins that eventually are  
10 secreted as a mature homodimer after cleavage of the signal peptide and pro-domain (*see e.g.* Rattenholl, et al., 2000, *J. Mol. Biol.*, 305:523; Fairlie et al., 2001, *J. Biol. Chem.*, 276(20):16911). Both the signal peptide and pro-domain mediate proper secretion for TGF beta family members (Rattenholl et al., 2000, *J. Mol. Biol.*, 305:523; Rattenholl et al., 2001, *Eur. J. Biochem.*, 268:3296).

#### Summary

The invention is based, at least in part, on the discovery that certain buffer compositions are particularly effective at inducing the refolding of a denatured polypeptide. The compositions and methods detailed herein were developed to induce  
20 protein refolding, so as to result in a polypeptide having a proper three dimensional structure and accompanying biological activity.

In one aspect, the invention features a method of inducing folding of a denatured polypeptide by: (1) providing a denatured polypeptide; and (2) contacting the polypeptide with an amount of a refolding buffer effective to induce folding of the  
25 polypeptide, wherein the refolding buffer contains (i) potassium phosphate or sodium phosphate at a concentration of 25 mM to 150 mM with a pH of 5.8 to 8.0, (ii) guanidine-HCl at a concentration of 0.3 M to 2 M, (iii) L-Arginine at a concentration of 0.25 M to 1 M, (iv) Tween-80 at a concentration of 0.05% to 1%, and (v) oxidized glutathione at a concentration of 1 mM to 4 mM and reduced glutathione at a  
30 concentration of 0.05 mM to 0.8 mM, wherein the ratio of oxidized to reduced glutathione is from 5:1 to 20:1.

In some embodiments, the denatured polypeptide is a polypeptide containing a TGF beta superfamily member.

“TGF beta superfamily member,” as used herein, refers to a protein having a sequence identical to a wild type member of the TGF beta superfamily, a truncate that  
5 retains the biological activity of the wild type protein, or a variant that has at least 70% sequence identity to the wild type protein (full length or mature protein) and retains the biological activity of the wild type protein. Members of the TGF beta superfamily, include, for example, TGF-betas, growth differentiation factors, bone morphogenetic proteins, activins, inhibins, and glial cell line-derived neurotrophic factors. In some  
10 embodiments, a variant has at least 70%, 80%, 85%, 90%, 95%, or 98% sequence identity to the full length wild type protein and retains the biological activity of the wild type protein. In some embodiments, a variant has at least 70%, 80%, 85%, 90%, 95%, or 98% sequence identity to the mature wild type protein and retains the biological activity of the wild type protein.

15 A description of the concentration of “refolding buffer” components used in the methods described herein refers to the final concentration of the refolding buffer components present in the reaction with the denatured polypeptide (not to the concentration of the components in a stock solution of refolding buffer prior to addition with other components of the folding reaction).

20 As used herein, “to induce folding of a polypeptide” refers to the induction of a tertiary structure in a polypeptide, and the acquisition of associated biological activity, that corresponds to that of the wild type protein.

The TGF beta superfamily member can be a glial cell line-derived neurotrophic factor (GDNF) family member. “GDNF family member,” as used herein, refers to a  
25 protein having a sequence identical to a wild type member of the GDNF family, a truncate that retains the biological activity of the wild type protein, or a variant that has at least 70% sequence identity to the wild type protein (full length or mature protein) and retains the biological activity of the wild type protein. Members of the GDNF family include GDNF, neurturin, neublastin, and persephin. In some embodiments, a variant has  
30 at least 70%, 80%, 85%, 90%, 95%, or 98% sequence identity to the full length wild type protein and retains the biological activity of the wild type protein. In some embodiments,

a variant has at least 70%, 80%, 85%, 90%, 95%, or 98% sequence identity to the mature wild type protein and retains the biological activity of the wild type protein.

In some embodiments, the GDNF family member is a neublastin protein. "A neublastin protein," as used herein, refers to a protein having a sequence identical to a wild type neublastin (e.g., human neublastin), a truncate that retains the biological activity of the wild type protein, or a variant that has at least 70% sequence identity to the wild type protein (full length or mature neublastin protein) and retains the biological activity of the wild type protein. In some embodiments, a variant has at least 70%, 80%, 85%, 90%, 95%, or 98% sequence identity to the full length wild type protein and retains the biological activity of the wild type protein. In some embodiments, a variant has at least 70%, 80%, 85%, 90%, 95%, or 98% sequence identity to the mature wild type protein (e.g., amino acid residues 108-220 of SEQ ID NO:1) and retains the biological activity of the wild type protein. A neublastin protein can, for example, contain or consist of amino acid residues 122-220 of SEQ ID NO:1, amino acid residues 117-220 of SEQ ID NO:1, or amino acid residues 108-220 of SEQ ID NO:1.

The method can further include expressing the polypeptide in bacteria (e.g., *E. coli*) prior to inducing folding with the refolding buffer. In some embodiments, the polypeptide is expressed in bacteria in an insoluble form and, prior to inducing folding with the refolding buffer, the insoluble polypeptide is contacted with an amount of a solubilization buffer effective to denature the polypeptide.

In some embodiments, the refolding buffer contains L-Arginine at a concentration of 0.30 M to 0.5M. In other embodiments, the refolding buffer contains L-Arginine at a concentration of at least 0.30 M. In other embodiments, the refolding buffer contains L-Arginine at a concentration of at least 0.35 M. In other embodiments, the refolding buffer contains L-Arginine at a concentration of 0.35 M.

In some embodiments, the refolding buffer contains Tween-80 at a concentration of 0.1% to 1%. In other embodiments, the refolding buffer contains Tween-80 at a concentration of 0.1% to 0.5%. In other embodiments, the refolding buffer contains Tween-80 at a concentration of at least 0.1%. In other embodiments, the refolding buffer contains Tween-80 at a concentration of 0.1%.

In some embodiments, the refolding buffer contains oxidized and reduced glutathione at a ratio of from 5:1 to 10:1. In other embodiments, the refolding buffer contains oxidized and reduced glutathione at a ratio of 5:1. In some embodiments, the refolding buffer contains oxidized glutathione at a concentration of 1 mM to 2 mM. In other embodiments, the refolding buffer contains oxidized glutathione at a concentration of 1 mM.

In some embodiments, the refolding buffer contains guanidine-HCl at a concentration of 0.5 M to 1.0 M. In other embodiments, the refolding buffer contains guanidine-HCl at a concentration of at least 0.5 M. In other embodiments, the refolding buffer contains guanidine-HCl at a concentration of 0.5 M.

In some embodiments, the refolding buffer contains potassium phosphate at a concentration of 25 mM to 100 mM. In other embodiments, the refolding buffer contains potassium phosphate at a concentration of 25 mM to 75 mM. In other embodiments, the refolding buffer contains potassium phosphate at a concentration of at least 50 mM. In other embodiments, the refolding buffer contains potassium phosphate at a concentration of 50 mM. In some embodiments, the refolding buffer contains potassium phosphate at a pH of 7.0 to 8.0. In other embodiments, the refolding buffer contains potassium phosphate at a pH of 7.5 to 8.0. In other embodiments, the refolding buffer contains potassium phosphate at a pH of about 7.8.

The refolding buffer can optionally contain or consist of the following components (i) potassium phosphate pH 7.8 at a concentration of 50 mM, (ii) guanidine-HCl at a concentration of 0.5 M, (iii) L-Arginine at a concentration of 0.35 M, (iv) Tween-80 at a concentration of 0.1%, (v) oxidized glutathione at a concentration of 1 mM, and (vi) reduced glutathione at a concentration of 0.2 mM.

In some embodiments, the refolding buffer does not consist of (i) potassium phosphate pH 7.8 at a concentration of 50 mM, (ii) guanidine-HCl at a concentration of 0.5 M, (iii) L-Arginine at a concentration of 0.35 M, (iv) Tween-80 at a concentration of 0.1%, (v) oxidized glutathione at a concentration of 1 mM, and (vi) reduced glutathione at a concentration of 0.2 mM.

In some embodiments, the refolding buffer lacks urea and/or glycine.

In an other aspect, the invention features a composition containing an amount of a refolding buffer effective to, when diluted by a factor of 1 to 10, induce folding of a neublastin polypeptide, wherein the refolding buffer contains the following components at 1 to 10 times the stated concentrations: (i) potassium phosphate or sodium phosphate at a concentration of 25 mM to 150 mM with a pH ranging from 5.8 to 8.0; (ii) guanidine-HCl at a concentration of 0.3 M to 2 M; (iii) L-Arginine at a concentration of 0.25 M to 1 M; (iv) Tween-80 at a concentration of 0.05% to 1%; and (v) oxidized glutathione at a concentration of 1 mM to 4 mM and reduced glutathione at a concentration of 0.05 mM to 0.8 mM, wherein the ratio of oxidized to reduced glutathione is from 5:1 to 20:1. Such a composition can optionally be used as a stock solution that is diluted with other components prior to commencement of a folding reaction.

In some embodiments, the refolding buffer contains L-Arginine at 1 to 10 times a concentration of 0.30 M to 0.5M. In other embodiments, the refolding buffer contains L-Arginine at 1 to 10 times a concentration of at least 0.30 M. In other embodiments, the refolding buffer contains L-Arginine at 1 to 10 times a concentration of at least 0.35 M. In other embodiments, the refolding buffer contains L-Arginine at 1 to 10 times a concentration of 0.35 M.

In some embodiments, the refolding buffer contains Tween-80 at 1 to 10 times a concentration of 0.1% to 1%. In other embodiments, the refolding buffer contains Tween-80 at 1 to 10 times a concentration of 0.1% to 0.5%. In other embodiments, the refolding buffer contains Tween-80 at 1 to 10 times a concentration of at least 0.1%. In other embodiments, the refolding buffer contains Tween-80 at 1 to 10 times a concentration of 0.1%.

In some embodiments, the refolding buffer contains oxidized and reduced glutathione at a ratio of from 5:1 to 10:1. In other embodiments, the refolding buffer contains oxidized and reduced glutathione at a ratio of 5:1. In some embodiments, the refolding buffer contains oxidized glutathione at 1 to 10 times a concentration of 1 mM to 2 mM. In other embodiments, the refolding buffer contains oxidized glutathione at 1 to 10 times a concentration of 1 mM.

In some embodiments, the refolding buffer contains guanidine-HCl at 1 to 10 times a concentration of 0.5 M to 1.0 M. In other embodiments, the refolding buffer contains guanidine-HCl at 1 to 10 times a concentration of at least 0.5 M. In other embodiments, the refolding buffer contains guanidine-HCl at 1 to 10 times a concentration of 0.5 M.

In some embodiments, the refolding buffer contains potassium phosphate at 1 to 10 times a concentration of 25 mM to 100 mM. In other embodiments, the refolding buffer contains potassium phosphate at 1 to 10 times a concentration of 25 mM to 75 mM. In other embodiments, the refolding buffer contains potassium phosphate at 1 to 10 times a concentration of at least 50 mM. In other embodiments, the refolding buffer contains potassium phosphate at 1 to 10 times a concentration of 50 mM. In some embodiments, the refolding buffer contains potassium phosphate at a pH of 7.0 to 8.0. In other embodiments, the refolding buffer contains potassium phosphate at a pH of 7.5 to 8.0. In other embodiments, the refolding buffer contains potassium phosphate at a pH of about 7.8.

The refolding buffer can optionally contain or consist of the following components at 1 to 10 times the stated concentrations: (i) potassium phosphate pH 7.8 at a concentration of 50 mM; (ii) guanidine-HCl at a concentration of 0.5 M; (iii) L-Arginine at a concentration of 0.35 M; (iv) Tween-80 at a concentration of 0.1%; (v) oxidized glutathione at a concentration of 1 mM; and (vi) reduced glutathione at a concentration of 0.2 mM.

In some embodiments, the refolding buffer does not consist of (i) potassium phosphate pH 7.8 at a concentration of 50 mM, (ii) guanidine-HCl at a concentration of 0.5 M, (iii) L-Arginine at a concentration of 0.35 M, (iv) Tween-80 at a concentration of 0.1%, (v) oxidized glutathione at a concentration of 1 mM, and (vi) reduced glutathione at a concentration of 0.2 mM.

In some embodiments, the refolding buffer lacks urea and/or glycine.

The compositions and methods described herein are advantageous in that they allow for the refolding and purification of large quantities of a properly refolded TGF beta superfamily protein, such as neublastin, in circumstances where the protein is



produced in a host (e.g., bacteria) that does not yield a properly folded, biologically active product.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the exemplary methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

#### Brief Description of the Drawings

Fig. 1 depicts the sequences of human and rat 113 amino acid and 104 amino acid forms of neublastin.

Fig 2. is a graph depicting absorbance detected following the incubation of solubilized neublastin of the refolding buffers detailed in Table 1 (buffer 4, which contains Tween-80 at a concentration of 1%, is not shown).

#### Detailed Description

The present invention provides compositions and methods for inducing folding of a denatured polypeptide belonging to the TGF beta superfamily. Application of certain compositions to induce the folding of denatured neublastin, a member of the TGF beta superfamily and the GDNF subfamily, is described in the accompanying working examples. Because neublastin has a cysteine knot structure common to members of the TGF beta superfamily and the GDNF subfamily, the refolding buffers described herein are expected to be effective at inducing the folding of other polypeptides belonging to the TGF beta superfamily and the GDNF subfamily.

Neublastin

The native human pre pro neublastin polypeptide is 220 amino acids long and has the following sequence: MELGLGGLSTLSHCPWPRRQPALWPTLAALALLSSVAEA  
SLGSAPRSPAPREGPPPVLASPAGHLPGGRTARWCSGRARRPPPQPSRPAPPPPAP  
 5 PSALPRGGRAARAGGPGSRARAAGARGCRLRSQ LVPVRA LGLGHR SDELVRFRF  
CSGSCRRARSPHDLSLASLLGAGALRPPPGSRPVSQPCCRPTRYEAVSFMDVNST  
WRTVDRLSATA CGCLG (SEQ ID NO:1).

The human neublastin signal peptide begins with the methionine at position 1 (underlined) and ends with alanine at position 39 (underlined). The full length pro-  
 10 domain of human neublastin begins with serine at position 40 (underlined) and ends with arginine at position 107 (underlined). Mature human neublastin polypeptide consists of the carboxy terminal 113 amino acids, beginning with alanine at position 108 and ending with glycine at position 220. The compositions and methods described herein provide for efficient folding of a denatured neublastin protein, including full length neublastin, a  
 15 mature neublastin (lacking the signal peptide and pro domains), or a biologically active truncate or variant of a mature neublastin.

A neublastin protein folded according to the methods described herein can vary in length. Although the mature human neublastin polypeptide can consist of the carboxy terminal 113 amino acids of pre pro neublastin, not all of the 113 amino acids are  
 20 required to achieve useful neublastin biological activity. Amino terminal truncation is permissible. Thus, a neublastin polypeptide can correspond to the carboxy terminal 99-113 amino acids of native human neublastin (i.e., its length can be 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, or 113 amino acids). Neublastin polypeptides consisting of the carboxy terminal 104 and 113 amino acids of neublastin  
 25 are exemplified in the working examples provided below.

In addition to varying in length, the neublastin polypeptide can vary in sequence. In particular, certain amino acid substitutions can be introduced into the neublastin sequence without appreciable loss of a neublastin biological activity described herein. In exemplary embodiments, a polypeptide can be at least 70%, 80%, 85%, 90%, 95%, 98%  
 30 or 99% identical to SEQ ID NO:1 (or 70%, 80%, 85%, 90%, 95%, 98% or 99% identical to amino acids 108-220 of SEQ ID NO:1). A variant neublastin polypeptide differing in

sequence from those disclosed in SEQ ID NO:1 (or amino acids 108-220 of SEQ ID NO:1) may include one or more conservative amino acid substitutions, one or more non conservative amino acid substitutions, and/or one or more deletions or insertions. In some embodiments, the variant neublastin polypeptide includes at least one amino acid substitution with respect to SEQ ID NO:1 (or amino acids 108-220 of SEQ ID NO:1), which provides an internal polymer conjugation site to which a polymer (e.g., a polyalkylene glycol moiety such as a polyethylene glycol moiety) can be conjugated (exemplary neublastin variants are described in WO 02/060929, the content of which is incorporated herein by reference). In some embodiments, the variant neublastin polypeptide includes at least one amino acid substitution (e.g., a non-conservative substitution) with respect to SEQ ID NO:1 (or amino acids 108-220 of SEQ ID NO:1), which decreases heparin binding (e.g., R155E, R156E, R158E, or R155,156E, or one or more of these substitutions at the corresponding position or positions in a mature neublastin polypeptide).

Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics such as substitutions within the following groups: valine, alanine and glycine; leucine, valine, and isoleucine; aspartic acid and glutamic acid; asparagine and glutamine; serine, cysteine, and threonine; lysine and arginine; and phenylalanine and tyrosine. The non polar hydrophobic amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Any substitution of one member of the above-mentioned polar, basic or acidic groups by another member of the same group can be deemed a conservative substitution.

A polypeptide used in the methods described herein can contain heterologous amino acid sequences in addition to a neublastin protein. "Heterologous," as used when referring to an amino acid sequence, means a sequence that originates from a source foreign to the particular host cell, or, if from the same host cell, is modified from its original form. Exemplary heterologous sequences include a heterologous signal sequence (e.g., native rat albumin signal sequence, a modified rat signal sequence, or a

human growth hormone signal sequence) or a sequence used for purification of a neublastin protein (e.g., a histidine tag).

#### Neublastin Activity

Neublastin polypeptides used in the methods described herein display at least one biological activity of native neublastin. A biologically active neublastin polypeptide is a polypeptide that, when dimerized, can bind, along with GFR $\alpha$ 3, to RET and induce RET dimerization and autophosphorylation. (See e.g. Sanicola et al., 1997, *Proc. Natl. Acad. Sci. USA*, 94:6238). Any method of determining receptor binding and receptor autophosphorylation can be used to evaluate the biological activity the neublastin polypeptide. For example, the KIRA assay described in Example 3 can be used to assess neublastin biological activity. (See also, Sadick et al., 1996, *Anal. Biochem.*, 235(2):207).

#### Refolding Buffer

In general, the refolding buffer used in the methods described herein includes the following components: (i) potassium phosphate at a concentration of 25 mM to 150 mM; (ii) guanidine-HCl at a concentration of 0.3 M to 2 M; (iii) L-Arginine at a concentration of 0.25 M to 1 M; (iv) Tween-80 at a concentration of 0.05% to 1%; and (v) oxidized glutathione at a concentration of 1 mM to 4 mM and reduced glutathione at a concentration of 0.05 mM to 0.8 mM, wherein the ratio of oxidized to reduced glutathione is from 5:1 to 20:1.

In some embodiments, sodium phosphate at a concentration of 25 mM to 150 mM can be used in place of potassium phosphate. The pH of the sodium phosphate or potassium phosphate used in these methods generally falls in the range of 5.8 to 8.0. In addition, in some embodiments, detergents such as Tween-20 or NP40 can be used in place of Tween-80 at a concentration of 0.05% to 1%.

The effectiveness of a particular refolding buffer at inducing folding of a denatured polypeptide can be evaluated by measuring the absorbance (OD 320) following the incubation of the denatured polypeptide in a particular buffer (see Example 1 and Fig. 2). Absorbance detected in such an assay indicates the presence of precipitated,

improperly folded protein. As depicted in Example 2 in the accompanying examples, a low absorbance reading indicates that a buffer is effective at inducing folding of a denatured polypeptide. Biological activity of the folded polypeptide can also be measured by the *in vitro* and/or *in vivo* biological assays described herein.

5

The following are examples of the practice of the invention. They are not to be construed as limiting the scope of the invention in any way.

### Examples

10

#### Example 1: Identification of a Neublastin Refolding Buffer

Recombinant neublastin was expressed as a 10 histidine-tagged fusion protein (Fig. 1) in *E. coli* under the control of a T7 promoter. Both human and rat 113 and 104 amino acid forms were derived from their respective constructs (Fig. 1) and were  
15 refolded and purified by the methods described herein. The starts of the 113 amino acid and the 104 amino acid forms are each underlined and in boldface text in Fig. 1.

When expressed in *E. coli*, neublastin is contained as an insoluble protein within inclusion bodies (IB). Therefore, neublastin must be isolated from IBs and refolded to obtain a soluble and bioactive product. Inclusion bodies were obtained by lysing *E. coli*  
20 expressing neublastin in PBS using a Gaulin press followed by centrifugation. Unless otherwise noted, all centrifugations were performed at 4°C, while all other steps were carried out at room temperature. To obtain the maximum possible yield of properly refolded neublastin, it is advantageous to start with IB pellets that are free of cell debris. To accomplish this, IB pellets were weighed and subjected to further washing in IB wash  
25 buffer (20 mM Tris pH 8.5 and 0.5 M EDTA; 8 ml per gram protein). The IB pellet was collected by centrifugation at 15,000 x g for 20 minutes, the cloudy supernatant discarded, and washed again in the same buffer containing 2% Triton-X 100 (8 ml per gram protein) to help remove contaminating lipids. A final wash was performed to  
30 remove the Tween-80 using wash buffer without detergent (8 ml per gram protein) and the supernatant again was discarded.

A freshly made solubilization buffer (6M guanidine-HCl, 0.05 M potassium phosphate pH 7.8, 0.1 M DTT, and 1.0 mM EDTA) was added to the pellet and mixed well using a polytron mixer. To ensure complete solubilization, the mixture was stirred over night at room temperature. The next day, the solution was clarified by

5 centrifugation at 10,000 rpm for 20 minutes. The supernatant was decanted into a new container, and the remaining insoluble pellet was weighed to allow estimation of recovery. Not all of the protein was solubilized by this process. At this point, the soluble protein was quantitated using a standard Bradford protein assay with BSA in solubilization as a control.

10 To determine whether certain buffer conditions might result in a high yield of properly refolded neublastin, an array of potential refolding buffers was prepared in a 96 well plate (see Table 1).

Table 1: 96-Well Plate Refolding Buffer Map

		A	B	C	D	E	F	G	H	I
		mM	mM	mM	mM	mM	mM	mM	mM	mM
1	Phos (pH7.8)	50	50	50	50	50	50	50	50	50
	Guanidine	500	500	500	500	500	500	500	500	500
	Arginine	150	250	350	150	250	350	150	250	350
	Glutathione Reduced	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Glutathione Oxidized	1	2	4	1	2	4	1	2	4
	Tween-80	0	0	0	0	0	0	0	0	0
2	Phos (pH7.8)	50	50	50	50	50	50	50	50	50
	Guanidine	500	500	500	500	500	500	500	500	500
	Arginine	150	250	350	150	250	350	150	250	350
	Glutathione Reduced	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Glutathione Oxidized	1	2	4	1	2	4	1	2	4
	Tween-80	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
3	Phos (pH7.8)	50	50	50	50	50	50	50	50	50
	Guanidine	500	500	500	500	500	500	500	500	500
	Arginine	150	250	350	150	250	350	150	250	350
	Glutathione Reduced	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Glutathione Oxidized	1	2	4	1	2	4	1	2	4
	Tween-80	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
4	Phos (pH7.8)	50	50	50	50	50	50	50	50	50
	Guanidine	500	500	500	500	500	500	500	500	500
	Arginine	150	250	350	150	250	350	150	250	350
	Glutathione Reduced	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Glutathione Oxidized	1	2	4	1	2	4	1	2	4
	Tween-80	1	1	1	1	1	1	1	1	1

As shown in Table 1, Guanidine HCl (0.5 M), reduced glutathione (0.2 mM), and potassium phosphate pH 7.8 (50 mM) were held constant throughout the plate, whereas the concentrations of L-Arginine, oxidized glutathione, and Tween-80 were varied.

5 L-Arginine was varied from 0.15 M to 0.35 M (addition of up to 0.8 M L-Arginine worked as well) while oxidized glutathione was varied from 1 to 4 mM. In addition, Tween-80 was varied from 0 to 1%. Because glycine in some cases can substitute for L-Arginine during refolding, a separate plate was prepared that kept all the buffer components the same with the exception of L-Arginine, which was substituted with  
10 glycine ranging from 25 to 100 mM.

The final volume of the buffer in each well was 280  $\mu$ l (reduced glutathione was added fresh from a stock concentration). Twenty microliters of solubilized neublastin was then added to each well at a final concentration of 0.1 mg/ml. The absorbance was monitored over a 48-hour period. Any detected absorbance indicated the presence of  
15 precipitated and not properly refolded protein.

The most occurrence of precipitation was observed with wells containing 0.15 M L-Arginine while the least amount of precipitation was observed in wells containing 0.35 M L-Arginine (Fig. 2). Of the wells containing 0.35 M L-Arginine, the best overall results were observed in those wells containing 0.1 % Tween-80. The best refolding was  
20 observed when a ratio of oxidized to reduced glutathione was 20:1 (but a 5:1 ratio was selected for the further experiments described herein so as to decrease the amount of oxidized glutathione needed in the refolding buffer). Based on these criteria, the refolding buffer system presented in the following examples was used and has provided high yield and properly refolded neublastin. Under all buffer conditions, the replacement  
25 of L-Arginine with glycine resulted in neublastin precipitation.

#### Example 2: Refolding and Purification of Neublastin

The results of the buffer analysis described in Example 1 were applied to prepare the following refolding buffer used in this and the following example: 0.5 M guanidine-  
30 HCl, 0.35 M L-Arginine, 50 mM potassium phosphate pH 7.8, 0.2 mM reduced glutathione, 1 mM oxidized glutathione, and 0.1% Tween-80. The refolding buffer was

made fresh. Solubilized protein was rapidly diluted into refolding buffer at a final protein concentration of 0.05 to 0.5 mg/ml. On average, 0.1 mg/ml of solubilized neublastin was used. This mixture was incubated at room temperature for at least 48 hours. No stirring was necessary.

5

#### Host cell contaminant removal using Ni-IMAC chromatography

L-Arginine was diluted from 0.35 M to 0.175 M to avoid leaching of Ni from the IMAC resin. This can be performed using either of the following methods. Arginine can be directly diluted to the proper concentration using 0.5 M guanidine-HCl. Water alone  
10 was not used because neublastin may precipitate if the guanidine concentration is not maintained (guanidine-HCl should be maintained in the buffers until the cationic chromatography step described below), resulting in a major loss in product recovery. Since directly diluting the L-Arginine would substantially increase the working volume and increase the amount of guanidine required, the protein was concentrated to 1/20<sup>th</sup> of  
15 the original volume using a Millipore tangential flow Pellicon unit. Following concentration, L-Arginine was diluted to 0.175 M using 0.5 M guanidine.

The L-Arginine diluted solution was applied to a Ni-NTA IMAC column that was previously equilibrated in column wash buffer (40 mM imidazole and 0.5 M guanidine HCl) using a flow rate of 50 to 100 ml per minute. Neublastin bound to the Ni-NTA  
20 matrix via the histidine tag and no product was observed in the flow through. Following washing with five column volumes of wash buffer, neublastin was eluted from the resin using 0.2 M imidazole in 0.5 M guanidine. The column wash buffer (which did not contain neublastin) was discarded. Protein recovery was monitored using a Bradford assay. In addition, host cell contaminants were monitored from this point onward.

25

#### Histidine tag separation from neublastin by protease digestion

One of two possible histidine tag removal procedures was employed, depending on the length of neublastin required (113 amino acids or 104 amino acids).

To generate the wild-type 113 amino acid neublastin product, Endo Lys C was  
30 used to clip the tag c-terminal of the lysine residue contained within the tag. Five units of Endo Lys C (WAKO, catalogue # 129-02541) per gram of neublastin were added to the



material from the Ni-NTA elution. No buffer substitution or pH adjustment was necessary (in some cases buffer substitution using 10 mM Hepes pH 7.8 was used and worked effectively). Neublastin with protease was incubated over night at room temperature with constant stirring.

5           To generate the 104 amino acid form of neublastin, the histidine-tagged product was treated with trypsin (Cooper Biomedical #3740) using a 1:2000 ratio of trypsin to neublastin. Again, no buffer substitution or pH adjustment was necessary. The mixture was incubated over night at room temperature with constant stirring.

10           Ni-NTA resin was equilibrated with wash buffer (0.5 M guanidine-HCl and 0.04 M imidazole). Following adjustment of the imidazole concentration within the neublastin preparation to 0.04 M from 0.2 M using 0.5 M guanidine-HCl, the material was applied to the Ni-NTA resin with a 50 to 100 ml per minute flow rate. The column flow through which contained non-tagged neublastin was collected and monitored for neublastin using the Bradford assay. To re-generate the Ni-NTA resin, the histidine tag  
15           was eluted using 0.2 M imidazole in 0.5 M guanidine HCl. This material was subjected to SDS/PAGE along with the resin flow through to establish the efficiency of the protease digestion.

20           The Ni-NTA flow through from the previous step was adjusted to 0.35 M guanidine-HCl by the addition of ddH<sub>2</sub>O. Higher concentrations of guanidine may prevent neublastin from binding to the cationic matrix. A C-100 filter-binding cartridge (Sartorius, catalogue # C100X) was equilibrated with C-100 wash buffer (5 mM sodium phosphate pH 6.5 and 0.35 M NaCl).

25           SP-Sepharose (AmershamPharmacia) can substitute for C-100 membrane filters. However, C-100 was chosen due to its increased surface area compared to that of classical column chromatography. When purifying neublastin on SP-Sepharose, local aggregation of neublastin can be prevented by choosing a larger column diameter and/or lowering protein load. This prevents high local concentrations of neublastin which can contribute to tetramer formation and product precipitation, especially when using sodium phosphate buffer.

30           Neublastin in 0.35 M guanidine-HCl was applied to the C-100 filter at a flow rate of 50 to 100 ml per minute followed by extensive washing of the filter with C-100 wash

buffer. This step removes any remaining histidine tag, endotoxin, and neublastin monomer. Neublastin dimer was recovered by eluting the protein from the C-100 matrix using 5 mM sodium phosphate pH 6.5 and 1 M sodium chloride. The elution was monitored by UV absorption at 280 nm and the neublastin peak collected in one  
5 container.

#### Neublastin concentration and buffer substitution

Neublastin was concentrated by Millipore Biomax-10 tangential flow filtration and diafiltered with the same unit to 5 mM sodium phosphate pH 6.5 and 0.15 M sodium  
10 chloride with 5 diafiltration volumes. An effort was made to aim for 1.0 – 1.5 mg/ml final protein concentration, and not permit the concentration go above 2.0 mg/ml, otherwise neublastin may begin to precipitate in this formulation with a large protein loss. Once the product was concentrated to 1.0 mg/ml and formulated in 5 mM sodium phosphate pH 6.5 and 0.15 M sodium chloride, neublastin was aliquoted into convenient  
15 sizes and stored at –70°C until needed.

#### Example 3: Analytical Characterization of Neublastin

Purified neublastin described in Example 2 was subjected to various analytical tests to verify purity, primary amino acid sequence, bioactivity, and disulfide structural  
20 integrity.

#### SDS/PAGE estimation of purity and molecular weight

Samples, taken from each of the neublastin refolding/purification steps, were subjected to SDS/PAGE analysis through a 4 to 20% acrylamide gel under non-reducing  
25 conditions. The final neublastin product migrated as a reducible dimer of 24,000 Da with an estimated purity of >98%.

#### Mass Spectrometry of refolding rat neublastin

To estimate the purity and to determine mass of the product refolded, neublastin  
30 was subjected to mass spectrometry on a ZMD mass spectrometer. Neublastin was denatured in 8M urea and treated with DTT prior to analysis to reduce all disulfide bonds

and convert the dimer molecule into monomer. The major signal identified represents rat neublastin residues 10 to 113 suggesting the predominant species in the preparation is as expected. However, a major signal at 10991 Da was identified and is predicted to correspond to a Leucine deletion, and a signal at 11076 is predicted to be a small amount of an Arginine to Lysine substitution. The low level peaks correspond to oxidation, acetonitrile adducts and TFA adducts. A small amount of the 106 amino acid form of neublastin was also identified. No trypsin-associated peaks were identified.

#### Characterization of rat 104 amino acid neublastin by AspN peptide mapping

AspN peptide mapping was carried out on neublastin that was produced by trypsin digestion to remove the histidine tag. This batch was compared to several other neublastin preparations including the wild-type rat 113 amino acid, wild-type human 113 amino acid, human 104 amino acid forms. Results demonstrated that this batch was as predicted, with approximately 8% oxidation at Met92, 5% Leu61 deletion, low levels of Arg to Lys mutations and less than 1% deamidation at Asn95.

#### Disulfide analysis of rat 104 amino acid neublastin

Disulfide analysis was carried out on rat 104 amino acid neublastin. Wild-type rat 113 amino acid neublastin was run in parallel as a reference. Approximately 150  $\mu$ L of refolded and purified neublastin was used for disulfide mapping. Results demonstrated that all disulfide linkages in the two samples are comparable and as expected. The profile of the neublastin monomer is similar to that of the reference, except for the area under low-level peaks eluting just ahead of the main monomer peak. These earlier-eluting peaks are predicted to contain, in part, oxidized monomer and were not included in downstream mass mapping. Fractions containing disulfide-linked peptides were pooled and analyzed by MALDI-TOF mass spectrometry using DHB as the matrix. The data indicated that rat 104 amino acid neublastin following AspN/trypsin digestion is as predicted, and there is no evidence of mixed disulfide connectivity.

Assay of neublastin activity using the Kinase Receptor Activation-Enzyme-linked Immunosorbant

Neublastin activity was determined by its ability to stimulate *c-Ret* phosphorylation in NB41A3-mRL3 cells, an adherent murine neuroblastoma cell line that expresses Ret and GFRa3. NB41A3-mRL3 cells were plated in DMEM supplemented with 10 % FBS at  $2 \times 10^5$  cells per well in 24-well plates, and cultured for 18 hours at 37°C and 5 % CO<sub>2</sub>. Following removal of the media and a cell wash with 1 ml of PBS per well, the cells were stimulated with DMEM containing either 113 amino acid or 104 amino acid neublastin for 10 minutes at 37°C and 5 % CO<sub>2</sub>. To stop neublastin activity, the media was removed and the cells washed with PBS immediately before lysis with 10 mM Tris, pH 8.0, 0.5 % NP40, 0.2 % DOC, 50 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 mM PMSF. After a 1-hour incubation at 4°C, the lysates were agitated by repeated pipeting and transferred (0.25 ml per well) to a 96-well ELISA plate coated with anti-RET mAb (AA.GE7.3). The wells were blocked at room temperature for 1 hour with blocking buffer (TBST containing 1 % normal mouse serum and 3 % BSA) followed by six washes with TBST alone. Phosphorylated RET was detected by incubating (2 hours) the captured receptor with HRP-conjugated phosphotyrosine antibody (4G10; 0.2 µg per well). Following the incubation, the wells were washed six times with TBST, and the HRP activity detected at 450 nm with a colorimetric assay. The absorbance values from wells treated with lysate or with lysis buffer alone were measured, background corrected, and the data plotted as a function of the concentration of neublastin present in the activation mixture. Rat 104 amino acid neublastin was as active in the KIRA assay as was the positive 113 amino acid neublastin control demonstrating that the refolding/purification process yields biologically active product.

Endotoxin assay

Using the Limulus Amebocyte Lysate assay and manufacturer-suggested conditions (Bio\*Whittaker), endotoxin levels in each of the purification steps were determined. The vast majority of the endotoxin is removed during the first Ni-NTA wash step. Following the addition of trypsin, it was observed that the endotoxin level went up slightly which most likely is due to endotoxin in the trypsin preparation used. Washing

the C100 column with a large amount of wash buffer appears to be useful to remove remaining endotoxin. Endotoxin levels within the final product were well below maximum acceptable levels.

5    Host cell protein assay

Using an *E. coli* host cell protein assay kit from Cygnus Technologies and manufacturer-suggested conditions, host cell protein contamination was monitored in each of the purification steps. This kit is an ELISA-based assay that is sensitive down to 1 ng/ml host cell protein. As with the endotoxin result above, most of the host protein  
10   clearance occurs during the first Ni-NTA chromatography as well as during the C100 washing. Host cell protein was determined to be less than 0.0001% of the final product.

Trypsin clearance assay

Trypsin clearance was monitored using a fluorescence-based assay using N-T-  
15   BOC-GLN-ALA-ARG 7-AMIDO-4-Methylcoumarin HCl as substrate and was sensitive down to less than 40 ng/ml. Most, if not all, of the added trypsin was removed by the C100 flow through wash. The amount of trypsin remaining in the final product was less than 0.004% (below the level of sensitivity).

20   Histidine-tag detection ELISA

A histidine tag ELISA using an anti-polyhistidine antibody was developed to monitor histidine-tagged neublastin remaining in the final preparation. As expected, the majority of the histidine tag was found in the material prior to the first Ni-NTA and none was in the first Ni-NTA flow through, indicating that the majority of the histidine-tagged  
25   neublastin bound the Ni-NTA resin. This material eluted from the resin with the 0.2 M imidazole elution. The sensitivity of this assay was approximate 0.3 µg/ml, and the final amount of histidine-tagged neublastin identified in the final product was determined to be 0.12% of the total protein or 0.88 mg.

#### Host cell DNA detection assay

Clearance of host cell DNA was monitored using an assay that utilizes single-stranded DNA binding protein coupled to avidin in an ELISA-based sandwich assay. This assay was demonstrated to be sensitive to approximately 200 pg/ml of *E. coli* DNA.

- 5 Based on the single-stranded DNA binding assay, the final neublastin preparation was determined to have less than 0.0001% contaminating host cell DNA. As with other assays described above, both the first Ni-NTA chromatography step and the C100 wash step were most efficient at removing the DNA impurities within the starting material.

#### 10 Chronic Constriction Injury (CCI) rats treated with 104 amino acid neublastin

- Neublastin treated CCI rats displayed diminished tactile allodynia as compared to vehicle treated controls. The neublastin treated rats were able to withstand a greater force applied to the ipsilateral foot. Tactile allodynia was evaluated with von Frey Hairs applying the up-down method (Chaplan et al., 1994). Rats were tested at days 7, 10, 14, 15 17, and 21 for altered nociceptive thresholds. Shams (n = 3) did not display a different gram threshold during the testing period, while all CCI rats had a lower threshold for the applied von Frey Hairs compared to their baseline values. Neublastin-104 1mg/Kg (n=8) and 3 mg/Kg (n=7) treated rats were able to withstand an elevated threshold compared to the vehicle treated controls (n = 8). The force withstood by neublastin treated animals 20 was statically significant ( $p < .05$ ) at days 17 and 21 post-op CCI. Thermal hyperalgesia was attenuated in the neublastin treated CCI rats, with the 3 mg/Kg dose demonstrating a higher efficacy than the 1 mg/Kg dose at day 21 post-op. Thermal hyperalgesia was determined using a Hargreaves device to assess thermal withdrawal latency. Rats were tested at days 7, 10, 14, 17, and 21 for lowered paw withdrawal latencies. Shams (n = 3) 25 did not display altered paw withdrawal latency during the testing period, while all CCI rats had a shorter paw withdrawal latency compared to their baseline values. Neublastin-104 1mg/Kg (n=8) and 3 mg/Kg (n=7) were able to withstand longer application of the thermal stimulus compared to the vehicle treated controls (n = 8) at days 14, 16 and 21 following CCI induction. While the 104 amino acid neublastin 3 mg/Kg-treated rats 30 demonstrated a significantly higher latency on day 21 post-op compared to the 104 amino acid neublastin 1 mg/Kg treated rats, the duration of paw withdrawal latency by

neublastin treated animals was statically significant ( $p < .05$ ) at days 14, 17, and 21 post-op CCI.

CCI rats treated with Neublastin were able to apply more weight to the affected chronic constricted hindlimb as seen with the incapacitance test. Incapacitance was  
5 determined using an incapacitance meter to assess the weight distribution of each foot. At baseline, rats distributed equal weight between their feet, but following injury there was less weight applied to the ipsilateral foot. Shams ( $n = 4$ ) did not display altered weight distribution between their feet during the testing period, while all CCI rats applied less weight to the affected foot compared to their baseline values. 104 amino acid  
10 Neublastin 1mg/Kg ( $n = 8$ ) and 3 mg/Kg ( $n = 7$ ) applied more weight to the ipsilateral foot as compared to the vehicle treated controls ( $n = 8$ ). The incapacitance of the affected foot in Neublastin treated animals was statically significant ( $p < .05$ ) at days 14, 17, and 21 post-op CCI.

While there was not a statistically significant difference between the Neublastin  
15 and vehicle treated CCI rats on the cold allodynia test, the Neublastin-treated rats tended to have shorter durations on day 10. Cold allodynia was determined using a copper cold plate chilled to 4°C for a 5 minute testing period. Rats were tested at days 7, 10, 14, 17, and 21 for elevated paw withdrawal duration compared to their baseline values. At baseline, no animals reacted to the cold. Shams ( $n = 3$ ) did not display elevated paw  
20 withdrawal duration throughout the testing period, while both all CCI rats had increased paw withdrawal duration compared to their baseline values. 104 amino acid Neublastin 1 mg/Kg ( $n = 8$ ) and 3 mg/Kg ( $n = 7$ ) elevated the affected paw for a shorter period of time compared to the vehicle treated controls ( $n = 8$ ) at days 14, 17, and 21 following CCI induction, although the duration of paw withdrawal by Neublastin treated animals was not  
25 statically significant.

Other Embodiments

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other  
5 aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:



1. A method of inducing folding of a denatured polypeptide, the method comprising:

providing a denatured polypeptide comprising a transforming growth factor beta (TGF beta) superfamily member; and

5       contacting the polypeptide with an amount of a refolding buffer effective to induce folding of the polypeptide, wherein the refolding buffer comprises (i) potassium phosphate or sodium phosphate at a concentration of 25 mM to 150 mM with a pH of 5.8 to 8.0, (ii) guanidine-HCl at a concentration of 0.3 M to 2 M, (iii) L-Arginine at a concentration of 0.25 M to 1 M, (iv) Tween-80 at a concentration of 0.05% to 1%, and  
10       (v) oxidized glutathione at a concentration of 1 mM to 4 mM and reduced glutathione at a concentration of 0.05 mM to 0.8 mM, wherein the ratio of oxidized to reduced glutathione is from 5:1 to 20:1.

2. The method of claim 1, wherein the refolding buffer comprises L-Arginine at a  
15       concentration of 0.30 M to 0.5M.

3. The method of claim 1, wherein the refolding buffer comprises L-Arginine at a concentration of at least 0.30 M.

20       4. The method of claim 1, wherein the refolding buffer comprises L-Arginine at a concentration of at least 0.35 M.

5. The method of claim 1, wherein the refolding buffer comprises L-Arginine at a concentration of 0.35 M.

25       6. The method of any of claims 1-5, wherein the refolding buffer comprises Tween-80 at a concentration of 0.1% to 1%.

7. The method of any of claims 1-5, wherein the refolding buffer comprises  
30       Tween-80 at a concentration of 0.1% to 0.5%.

8. The method of any of claims 1-5, wherein the refolding buffer comprises Tween-80 at a concentration of at least 0.1%.

5 9. The method of any of claims 1-5, wherein the refolding buffer comprises Tween-80 at a concentration of 0.1%.

10. The method of any of claims 1-9, wherein the refolding buffer comprises oxidized and reduced glutathione at a ratio of from 5:1 to 10:1.

10 11. The method of any of claims 1-9, wherein the refolding buffer comprises oxidized and reduced glutathione at a ratio of 5:1.

12. The method of any of claims 1-9, wherein the refolding buffer comprises oxidized glutathione at a concentration of 1 mM to 2 mM.

15 13. The method of any of claims 1-9, wherein the refolding buffer comprises oxidized glutathione at a concentration of 1 mM.

14. The method of any of claims 1-13, wherein the refolding buffer comprises guanidine-HCl at a concentration of 0.5 M to 1.0 M.

15. The method of any of claims 1-13, wherein the refolding buffer comprises guanidine-HCl at a concentration of at least 0.5 M.

25 16. The method of any of claims 1-13, wherein the refolding buffer comprises guanidine-HCl at a concentration of 0.5 M.

17. The method of any of claims 1-16, wherein the refolding buffer comprises potassium phosphate at a concentration of 25 mM to 100 mM.

30

18. The method of any of claims 1-16, wherein the refolding buffer comprises potassium phosphate at a concentration of 25 mM to 75 mM.

19. The method of any of claims 1-16, wherein the refolding buffer comprises potassium phosphate at a concentration of at least 50 mM.

20. The method of any of claims 1-16, wherein the refolding buffer comprises potassium phosphate at a concentration of 50 mM.

21. The method of any of claims 1-20, wherein the refolding buffer comprises potassium phosphate at a pH of 7.0 to 8.0.

22. The method of any of claims 1-20, wherein the refolding buffer comprises potassium phosphate at a pH of 7.5 to 8.0.

23. The method of any of claims 1-20, wherein the refolding buffer comprises potassium phosphate at a pH of about 7.8.

24. The method of any of claims 1-23, wherein the refolding buffer does not consist of (i) potassium phosphate pH 7.8 at a concentration of 50 mM, (ii) guanidine-HCl at a concentration of 0.5 M, (iii) L-Arginine at a concentration of 0.35 M, (iv) Tween-80 at a concentration of 0.1%, (v) oxidized glutathione at a concentration of 1 mM, and (vi) reduced glutathione at a concentration of 0.2 mM.

25. The method of claim 1, wherein the refolding buffer comprises (i) potassium phosphate pH 7.8 at a concentration of 50 mM, (ii) guanidine-HCl at a concentration of 0.5 M, (iii) L-Arginine at a concentration of 0.35 M, (iv) Tween-80 at a concentration of 0.1%, (v) oxidized glutathione at a concentration of 1 mM, and (vi) reduced glutathione at a concentration of 0.2 mM.

26. The method of claim 1, wherein the refolding buffer consists of (i) potassium phosphate pH 7.8 at a concentration of 50 mM, (ii) guanidine-HCl at a concentration of 0.5 M, (iii) L-Arginine at a concentration of 0.35 M, (iv) Tween-80 at a concentration of 0.1%, (v) oxidized glutathione at a concentration of 1 mM, and (vi) reduced glutathione at a concentration of 0.2 mM.

27. The method of any of claims 1-23, wherein the refolding buffer lacks urea.

28. The method of any of claims 1-23, wherein the refolding buffer lacks glycine.

29. The method of any of claims 1-28, wherein the TGF beta superfamily member is a glial cell line-derived neurotrophic factor (GDNF) family member.

30. The method of claim 29, wherein the GDNF family member is a neublastin protein.

31. The method of claim 30, wherein the neublastin protein comprises amino acid residues 122-220 of SEQ ID NO:1.

32. The method of claim 30, wherein the neublastin protein comprises amino acid residues 117-220 of SEQ ID NO:1.

33. The method of claim 30, wherein the neublastin protein comprises amino acid residues 108-220 of SEQ ID NO:1.

34. The method of any of claims 1-33, further comprising expressing the polypeptide in bacteria prior to inducing folding with the refolding buffer.

35. The method of claim 34, wherein the bacteria is *E. coli*.

36. The method of claim 34 or 35, wherein the polypeptide is expressed in bacteria in an insoluble form and, prior to inducing folding with the refolding buffer, the insoluble polypeptide is contacted with an amount of a solubilization buffer effective to denature the polypeptide.

5

37. A composition comprising an amount of a refolding buffer effective to, when diluted by a factor of 1 to 10, induce folding of a neublastin polypeptide, wherein the refolding buffer comprises the following components at 1 to 10 times the stated concentrations: (i) potassium phosphate or sodium phosphate at a concentration of 25 mM to 150 mM with a pH ranging from 5.8 to 8.0; (ii) guanidine-HCl at a concentration of 0.3 M to 2 M; (iii) L-Arginine at a concentration of 0.25 M to 1 M; (iv) Tween-80 at a concentration of 0.05% to 1%; and (v) oxidized glutathione at a concentration of 1 mM to 4 mM and reduced glutathione at a concentration of 0.05 mM to 0.8 mM, wherein the ratio of oxidized to reduced glutathione is from 5:1 to 20:1.

15

38. The composition of claim 37, wherein the refolding buffer comprises L-Arginine at 1 to 10 times a concentration of 0.30 M to 0.5M.

39. The composition of claim 37, wherein the refolding buffer comprises L-Arginine at 1 to 10 times a concentration of at least 0.30 M.

40. The composition of claim 37, wherein the refolding buffer comprises L-Arginine at 1 to 10 times a concentration of at least 0.35 M.

41. The composition of claim 37, wherein the refolding buffer comprises L-Arginine at 1 to 10 times a concentration of 0.35 M.

42. The composition of any of claims 37-41, wherein the refolding buffer comprises Tween-80 at 1 to 10 times a concentration of 0.1% to 1%.

30

43. The composition of any of claims 37-41, wherein the refolding buffer comprises Tween-80 at 1 to 10 times a concentration of 0.1% to 0.5%.

5 44. The composition of any of claims 37-41, wherein the refolding buffer comprises Tween-80 at 1 to 10 times a concentration of at least 0.1%.

45. The composition of any of claims 37-41, wherein the refolding buffer comprises Tween-80 at 1 to 10 times a concentration of 0.1%.

10 46. The composition of any of claims 37-45, wherein the refolding buffer comprises oxidized and reduced glutathione at a ratio of from 5:1 to 10:1.

15 47. The composition of any of claims 37-45, wherein the refolding buffer comprises oxidized and reduced glutathione at a ratio of 5:1.

48. The composition of any of claims 37-45, wherein the refolding buffer comprises oxidized glutathione at 1 to 10 times a concentration of 1 mM to 2 mM.

20 49. The composition of any of claims 37-45, wherein the refolding buffer comprises oxidized glutathione at 1 to 10 times a concentration of 1 mM.

50. The composition of any of claims 37-49, wherein the refolding buffer comprises guanidine-HCl at 1 to 10 times a concentration of 0.5 M to 1.0 M.

25 51. The composition of any of claims 37-49, wherein the refolding buffer comprises guanidine-HCl at 1 to 10 times a concentration of at least 0.5 M.

30 52. The composition of any of claims 37-49, wherein the refolding buffer comprises guanidine-HCl at 1 to 10 times a concentration of 0.5 M.

53. The composition of any of claims 37-52, wherein the refolding buffer comprises potassium phosphate at 1 to 10 times a concentration of 25 mM to 100 mM.

54. The composition of any of claims 37-52, wherein the refolding buffer  
5 comprises potassium phosphate at 1 to 10 times a concentration of 25 mM to 75 mM.

55. The composition of any of claims 37-52, wherein the refolding buffer comprises potassium phosphate at 1 to 10 times a concentration of at least 50 mM.

10 56. The composition of any of claims 37-52, wherein the refolding buffer comprises potassium phosphate at 1 to 10 times a concentration of 50 mM.

57. The composition of any of claims 37-56, wherein the refolding buffer comprises potassium phosphate at a pH of 7.0 to 8.0.

15 58. The composition of any of claims 37-56, wherein the refolding buffer comprises potassium phosphate at a pH of 7.5 to 8.0.

59. The composition of any of claims 37-56, wherein the refolding buffer  
20 comprises potassium phosphate at a pH of about 7.8.

60. The composition of any of claims 37-59, wherein the refolding buffer does not consist of (i) potassium phosphate pH 7.8 at a concentration of 50 mM, (ii) guanidine-HCl at a concentration of 0.5 M, (iii) L-Arginine at a concentration of 0.35 M,  
25 (iv) Tween-80 at a concentration of 0.1%, (v) oxidized glutathione at a concentration of 1 mM, and (vi) reduced glutathione at a concentration of 0.2 mM.

61. The composition of claim 37, wherein the refolding buffer comprises the following components at 1 to 10 times the stated concentrations: (i) potassium phosphate  
30 pH 7.8 at a concentration of 50 mM; (ii) guanidine-HCl at a concentration of 0.5 M; (iii) L-Arginine at a concentration of 0.35 M; (iv) Tween-80 at a concentration of 0.1%;

(v) oxidized glutathione at a concentration of 1 mM; and (vi) reduced glutathione at a concentration of 0.2 mM.

5        62. The composition of claim 37, wherein the refolding buffer consists of the following components at 1 to 10 times the stated concentrations: (i) potassium phosphate pH 7.8 at a concentration of 50 mM; (ii) guanidine-HCl at a concentration of 0.5 M; (iii) L-Arginine at a concentration of 0.35 M; (iv) Tween-80 at a concentration of 0.1%; (v) oxidized glutathione at a concentration of 1 mM; and (vi) reduced glutathione at a concentration of 0.2 mM.

10

63. The composition of any of claims 37-59, wherein the refolding buffer lacks urea.

15        64. The composition of any of claims 37-59, wherein the refolding buffer lacks glycine.



Huneublastin	(1)	MGHHHHHHHHHSSGHIDDDDKAAGPGSRARAAGARGCRLRSQLPVPRAL
rneublastin	(1)	MGHHHHHHHHHSSGHIDDDDKAAGTRSSRARATDARGCRLRSQLPVSAL
Huneublastin	(51)	GLGHRSEDLVRFRCGSGCRRRARSPHDLSLASLLGAGALRPPPGSRPVSQ
rneublastin	(51)	GLGHSDELIRFRFCGSGCRRRARSPHDLSLASLLGAGALRSPPGSRPISQ
Huneublastin	(101)	PCCRPTRYEAVSFMDVNSTWRTVDRLSATAACGCLG (SEQ ID NO:2)
rneublastin	(101)	PCCRPTRYEAVSFMDVNSTWRTVDHLSATAACGCLG (SEQ ID NO:3)

Fig. 1

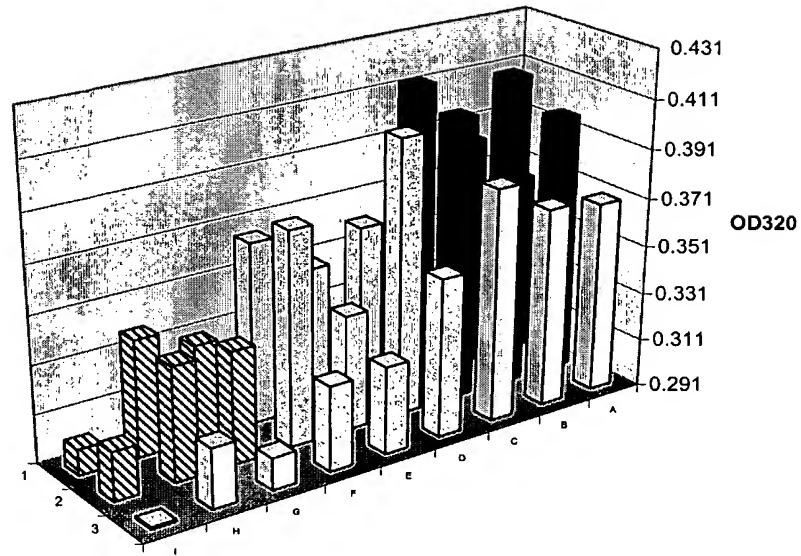


Fig. 2

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US05/29638

## A. CLASSIFICATION OF SUBJECT MATTER

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USPC: 514/12

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## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
USPAT, USOCR, PGPUBS, EPO, JPO, DERWENT, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2004/0028613 A1 (QUAY) 12 February 2004 (12.02.2004)	
A	US 2003/0186267 A1 (FEDER et al.) 02 October 2003 (02.10.2003)	
A	RIGANTI et al. Nitroarginine methyl ester and canavanine lower intracellular reduced glutathione. Free Radic Biol Med. 2003 Nov 15;35(10):1210-6	

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

### \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T"

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US05/29638

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 10-24, 27-36, 46-60, 63 and 64  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

- Remark on Protest**
- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
  - ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
  - ☐ No protest accompanied the payment of additional search fees.